Differential effects of fasting and leptin on proopiomelanocortin peptides in the arcuate nucleus and in the nucleus of the solitary tract

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Submitted 5 September 2006; accepted in final form 21 January 2007

Perello M, Stuart RC, Nillni EA. Differential effects of fasting and leptin on proopiomelanocortin peptides in the arcuate nucleus and in the nucleus of the solitary tract. Am J Physiol Endocrinol Metab 292: E1348–E1357, 2007. First published January 16, 2007; doi:10.1152/ajpendo.00466.2006.—The α-melanocyte-stimulating hormone (α-MSH), derived from proopiomelanocortin (POMC), is generated by a posttranslational processing mechanism involving the prohormone convertases (PCs) PC1/3 and PC2. In the brain, α-MSH is produced in the arcuate nucleus (ARC) of the hypothalamus and in the nucleus of the solitary tract (NTS) of the medulla. This peptide is key in controlling energy balance, as judged by changes observed at transcriptional level. However, little information is available regarding the biosynthesis of the precursor POMC and the production of its processed peptides during feeding, fasting, and fasting plus leptin in the ARC compared with the NTS in conjunction with the PC activity. In this study we found that, in the ARC, pomc mRNA, POMC-derived peptides, and PC1/3 all decreased during fasting, and administration of leptin reversed these effects. In contrast, in the NTS, where there is a large amount of a 28.1-kDa peptide similar in size to POMC, the 28.1-kDa peptide and other POMC-derived peptides, including α-MSH, were further accumulated in fasting conditions, whereas pomc mRNA decreased. These changes were not reversed by leptin. We also observed that, during fasting, PC2 levels decreased in the NTS. These data suggest that, in the NTS, fasting induced changes in POMC biosynthesis, and processing is independent of leptin. These observations indicate that changes in energy status affect POMC in the brain in a tissue-specific manner. This represents a novel aspect in the regulation of energy balance and may have implications in the pathophysiology of obesity.

Posttranslational processing of prohormones is an important biochemical mechanism employed by many secretory cells to generate a diversity of biological peptides from the same gene product, a process that occurs in a tissue-specific manner (33). Similarly to many prohormones, proopiomelanocortin (POMC) is subjected to sequence-specific cleavages to generate bioactive products by the action of the prohormone convertases (PCs) PC1/3 and PC2 (2). Thus far, the processing of POMC has been described in the pituitary and in the arcuate nucleus (ARC) of the hypothalamus. In the ARC, similar to the pars intermedia of the pituitary (1, 14, 19, 34), POMC is initially cleaved by PC1/3 to generate proadrenocorticotropic and β-lipotropin. Proadrenocorticotropic is further cleaved by PC1/3 to generate a 16-kDa NH2-terminal peptide and ACTH. ACTH is further cleaved to generate ACTH-(1–17) and corticotropin-like intermediate lobe peptide (CLIP). Then, carboxypeptidase E enzyme removes COOH-terminal basic amino acids from ACTH-(1–17), and the peptidyl α-amidating mono-oxygenase enzyme amidates the peptide to generate desacetyl α-melanocyte stimulating hormone (α-MSH). Finally, an N-acetylaspartylglutamate peptidase converts desacetyl α-MSH to acetyl α-MSH (7, 40, 50). POMC (4, 36) is also generated in the commissural part of the nucleus of the solitary tract (NTS). However, the fate of its processing and its role in energy balance is completely unknown.

The hormone leptin, produced in the adipocytes, directly activates POMC neurons in the ARC through the leptin receptor ObRb. Leptin induces the upregulation of the pomc gene and potentially the release of the α-MSH peptide from many neuronal projections to different regions of the brain inside and outside of the hypothalamus (11, 13, 44). In the paraventricular nucleus (PVN), for example, α-MSH released from POMC fibers originated in the ARC is the main regulator of the indirect pathway of leptin action controlling the hypothalamic pituitary thyroid axis (38). The anorectic role of α-MSH mediated by leptin has been extensively studied in the ARC of the hypothalamus (5, 43, 48) by examining pomc expression; however, limited information is available regarding regulation at the posttranslational processing and the secretion of POMC-derived peptides by leptin (11, 40).

POMC neurons in the NTS innervate different areas of the brain, but not the hypothalamus (11, 27, 39). The NTS, located within the brain stem, mediates some actions of leptin on energy balance. For example, administration of leptin in the fourth ventricle or directly in the dorsal vagal complex (DVC), which includes NTS, area postrema, and dorsal motor nucleus (DMX), inhibits food intake (21). Leptin treatment also increases c-Fos expression in some POMC neurons in the NTS (11); however, it is unknown whether leptin directly regulates POMC expression or processing in this nucleus.

In this study we investigated POMC gene expression, biosynthesis, and posttranslational level in the ARC compared with the NTS by using rats subjected to fasting and rats that were fasted and treated with leptin. Since we (42) recently demonstrated that leptin upregulates PC1/3 and PC2 expression in the PVN, we also determined whether a similar regulation of PC1/3 and PC2 expression occurred in the ARC and NTS. With a combination of gene expression, morphological, and biochemical studies in an in vivo system we demonstrated that the pomc gene, the processing of its protein precursor, and the PCs were regulated in a different manner in the ARC.
compared with the NTS in response to changes in energy homeostasis. Importantly, although leptin administration completely reversed the effects of fasting in the ARC, the NTS was not affected.

MATERIALS AND METHODS

Materials. Recombinant mouse leptin was obtained from Dr. Alan Parlow (Harbor-University of California at Los Angeles Medical Center). Dr. Nabil Seidah, from the Canada Research Institute of Montreal, donated the antibodies against PC1/3 and PC2. BioSource International (Camarillo, CA) generated the rabbit anti-α-MSH and rabbit anti-ACTH antisera using a customized acetyl α-MSH (acetyl-SYSMEHFRWGKPCV-amide) and the carboxy-terminal sequence of ACTH (KRPRVKVPNVAENESAEFPFLF) peptides, respectively. Synthetic acetyl α-MSH, ACTH, desacetyl α-MSH, and CLIP were obtained from American Peptide (Sunnyvale, CA). Except when indicated, all drugs were purchased from Sigma Aldrich (St. Louis, MO).

Animals, tissue dissections, and samples. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 175–200 g, were divided into three groups: fed, fasted, and fasted plus leptin. The first group was allowed free access to food. The second group was fasted for 65 h, beginning at 1600 on the first day and ending between 0900 and 1100 on the third day. The third group was fasted as described above, but 0.5 μg/g body wt of recombinant leptin was administered intraperitoneally every 6 h, beginning 2 h after the removal of food. All animals were allowed free access to water and were weighed daily. At the completion of the experiment, rats were killed by decapitation. The ARC- and NTS-enriched sections (hereafter referred to as NTS) were dissected out. The brain was removed from the skull, leaving the pituitary under the dura, and placed in a brain matrix (Kent Scientific, Torrington, CT), with the ventral surface on top under a dissecting microscope. To exclude possible contamination from the pars tuberalis of the pituitary, we transected the remaining pituitary stalk above the level of the median eminence (ME). To remove the hypothalamus, we used the optic chiasm and rostral edge of mammillary bodies as rostral and caudal limits, respectively; the hypothalamic sulci were used as lateral limits, and a 3-mm-thick slice was taken parallel to the base of the hypothalamus. We removed the ARC from the whole hypothalamus by cutting between the rostral and caudal limits of the ME and 0.5 mm to each lateral side of the ME. The depth of each section isolated was −1 mm thick, in parallel to the base of the hypothalamus. The NTS region was isolated as follows: the dorsal surface of medulla oblongata was exposed by removing the cerebellum, and a section of the dorsal caudal medulla just posterior to the obex was dissected for analysis. This section included the NTS as well as the dorsal vagal nucleus, the hypoglossal nucleus, the nucleus commissuralis, the nucleus gracilis, the nucleus cuneiformis, and the fasciculus cuneiformis but excluded the nucleus ambiguus, the nucleus tractus olfatorius lateralis, the tractus corticospinalis, the nucleus raphe magnus, and the nucleus reticularis lateralis (9). The ARC and NTS sections were collected in 600 μl of 2 N acetic acid freshly supplemented with a protease inhibitor mix (AEBSF, pepstatin A, E64, bestatin, leupeptin, and aprotinin) for POMC-derived peptides content analysis or in 800 μl of TRIzol Reagent (Invitrogen, La Jolla, CA) for RNA isolation. For brain fixation, fasted and fed adult male rats were deeply anesthetized with pentobarbital sodium (60 mg/kg ip), perfused transcardially with saline solution, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and postfixed in the same fixative for 2 h and cryoprotected by overnight immersion in a solution containing 20% sucrose in 0.1 M phosphate buffer. Twenty-five-micrometer tissue coronal sections were cut using a cryostat and used for immunohistochemistry. The Institutional Animal Care and Use Committee of Rhode Island Hospital/Brown University approved the experimental protocols and euthanasia procedures.
100% of acetyl and desacetyl α-MSH of the amidated forms, which are the ones detected in tissue samples analyzed. The cross-reactivity of the α-MSH assay with ACTH was ~16%; however, since the amount of ACTH detected in the ARC and NTS is below the ED_{50} of the assay, we were not able to see its presence in the HPLC samples. Using the α-MSH assay, we did not find any cross-reactivity with CLIP. The ACTH RIA assay was performed in 0.5 ml of RIA buffer containing anti-ACTH antiserum (1:30,000) and 5,000 rpm of 125I-ACTH tracer. The sensitivity of the assays was ~1.0 pg/tube, and the intra- and interassay variabilities were ~5–7 and 10–11%, respectively. The ACTH assay used under these experimental conditions can detect 100% of CLIP and ACTH forms; however, this assay does not cross-react with any form of α-MSH. The ACTH assay cross-reacts with the POMC precursor, as was demonstrated using a purified POMC (kindly donated by Dr. Anne White from the University of Manchester), and the α-MSH assay with the 28.1-kDa form was also seen in fraction no. 35 of the HPLC run.

### Purification and characterization of POMC peptides in the NTS

Fourteen male Sprague-Dawley rats were killed, and the NTS was removed and the presence of the peak in fraction no. 35. This fraction was reconstituted in water. One microliter of sample, containing 3.5 pg, was spotted on a normal-phase chip array (NP20; Ciphergen Biosystems, Fremont, CA) and allowed to dry. The NP20 array also received two applications of 1 µl of 20% saturated solution of a-cyano-4-hydroxycinnamic acid matrix (Ciphergen Biosystems) dissolved in acetonitrile and 1% TFA and allowed to dry between applications. The array was read using a Protein Chip System Series 4000 (Ciphergen Biosystems). Spectra of sample and calibrated standards were generated in the auto mode, using appropriate laser power. Analysis of the spectra was accomplished using Ciphergen Express Software (version 3.0) (28).

### Immunohistochemistry

Hypothalamic floating sections from fasted and fed animals were incubated with either anti α-MSH, anti-PC1/3, or anti-PC2 rabbit polyclonal antibodies. Briefly, sections were rinsed in 0.1 M Tris-buffered saline, pH 7.4, containing 0.05% Tween-20 and preincubated for 1 h in Tween-Tris-buffered saline (TTBS) containing 5% normal goat serum (NGS). Sections were then incubated for 48 h at 4°C in TTBS containing 0.5% NGS and 1:2,000 anti α-MSH, 1:1,000 anti-PC1/3, or 1:1,000 anti-PC2 antibodies. Sections were subsequently rinsed in TTBS and incubated for 1 h with Alexa fluor 594 goat anti-rabbit IgG ( Molecular Probes, Eugene, OR) diluted 1:500 in TTBS and 3% NGS. Sections were then rinsed in TTBS and embedded with Vectashield solution (Vector Laboratories, Burlingame, CA). Confocal images were acquired with a Nikon PCM 2000 (Nikon, Melville, NY), using the green Helium-Neon laser (543 nm). Serial optical sections were performed with Simple 3-C imaging computer software (Compix, Tualatin, OR). Z series sections were collected at 1 μm with an ×60 PlanApo lens or an ×20 Plan Apo lens. A scan zoom of ×1 was used in the acquisition of images. Images were processed and reconstructed in National Institutes of Health (NIH) Image shareware (NIH, Springfield, VA). Adobe Photoshop was used for the assembly of figures (Adobe System, San Jose, CA). All the semiquantitative measurements were carried out in comparable areas and under the same optical and light conditions. Fluorescence intensity was measured using Scion Image software (Scion, Frederick, MD). The units of optical density (OD) measured were calculated by the mean gray of the pixels inside the perimeter of the cell and expressed as arbitrary units of intensity (AU). The OD background of each section was subtracted from the OD measurement of each positive staining. A total of 20 images per animal and per condition were analyzed to construct the percentage analysis data, and each group had three animals.

### Statistical analysis

The results are presented as means ± SE. Statistical significance was determined by analysis of variance followed by post hoc Newman-Keuls test. Differences were considered to be significant at P < 0.05.

### RESULTS

The biosynthesis of POMC peptides in the hypothalamic ARC is regulated by leptin. Consistent with previous findings (5, 43) showing that leptin regulates pomc gene expression in the ARC, we found that the expression of proPOMC decreased from 1.00 ± 0.08 to 0.53 ± 0.03 in the fasted animals (P < 0.05 vs. control). Administration of leptin to fasted animals only partially reversed the effect of fasting on pomc expression to 0.70 ± 0.05 (Fig. 1A). Then we investigated whether changes in pomc gene expression observed during fasting were correlated with the biosynthesis of the protein. Analysis of ARC samples subjected to reverse-phase HPLC followed by the ACTH RIA revealed the presence of a major peak in fraction no. 30, which had the same elution profile as the synthetic ACTH (Fig. 1B). This antibody also recognized the CLIP peptide (fraction no. 28), which had the same elution profile as the synthetic CLIP. The peak eluted in fraction no. 35 most likely corresponds to POMC, as determined by the mass spectrometry analysis shown in Fig. 3C and consistent with its high hydrophobic sequence. It is important to point out that, whereas the ratio of ACTH to POMC in the ARC was slightly >5, in the whole hypothalamus this ratio was 32.7 (Fig. 1B, inset), demonstrating that a considerable amount of ACTH is distributed in neuronal fibers away from the ARC. Two other peaks eluted before CLIP were not yet identified. The data showed that the amount of ACTH (0.037 ± 0.004 fmol/μg protein) in the ARC represented ~4% of the total desacetyl α-MSH (1.001 ± 0.043 fmol/μg protein) generated. This indicates that, in this tissue, ACTH is mainly processed to desacetyl α-MSH, which is consistent with early studies by Emeson and Eipper (14) and other laboratories (15, 18, 49). Desacetyl α-MSH represented the major form of α-MSH in the ARC in fed rats (Fig. 1C), whereas acetyl α-MSH accounted for <10% of total α-MSH, and is normally close to the ED_{50} of our RIA standard curve. The smaller peaks eluted at fraction nos. 11 and 13 represent the sulfoxide forms of desacetyl α-MSH and acetyl α-MSH, respectively (Fig. 1C). Fasting decreased ACTH levels from 0.171 ± 0.019 to 0.086 ± 0.009 pg/μg protein (P < 0.05 vs. control), and administration of leptin increased the level of ACTH to 0.137 ± 0.014 pg/μg protein (Fig. 1B). This action of leptin on POMC peptides was also seen for all the peptides cross-reacting with this antibody, including POMC. These findings suggest that fasting, in addi-
tion to downregulating the pomc gene, also decreases the biosynthesis of its protein in the hypothalamus (Fig. 1B). Similarly, when we analyzed the same eluted fractions from fasted animals using the α-MSH RIA assay, we observed a decrease in desacetyl α-MSH levels from 1.63 ± 0.07 to 1.26 ± 0.04 pg/μg protein (P < 0.05 vs. control). Leptin administration increased the level of desacetyl α-MSH to 1.75 ± 0.11 pg/μg protein (Fig. 1C).

We then examined α-MSH, PC1/3, and PC2 protein expression in the fasted vs. fed condition using an immunohistochemistry protocol. The peptide α-MSH was distributed mostly in neuronal fibers (Fig. 2, A and B). As shown in the lower magnification in Fig. 2, A and B, insets, the region of the ARC also stained with this antibody. A comparison of the OD units (see MATERIALS AND METHODS) between fed and fasted did not show differences in expression. These results are not surprising because of the limitations of this semiquantitative analysis method of immunohistochemistry. Nevertheless, the biochemical data presented in Fig. 1C clearly showed a small but significant decrease of desacetyl α-MSH during fasting. Similarly to the PVN, fasting reduced the protein expression of PC1/3 (Fig. 2F). The ME region (see Fig. 2, D and E, insets) showed stronger immunostaining for PC1/3 in fed compared with fasted animals, consistent with our earlier report (42). The staining for PC2 was weaker than that observed for PC1/3, and no significant differences were observed between the fed and fasted animals (Fig. 2, G and H).

The biosynthesis of POMC peptides in the NTS is affected by fasting but is not regulated by leptin. We next wanted to determine the effect of fasting on POMC produced in the NTS
at the gene, translational, and posttranslational levels. As shown in Fig. 3A, we found that the \textit{pomc} gene expression decreased during fasting from 1.00 \pm 0.04 to 0.55 \pm 0.06 \((P < 0.05 \text{ vs. CTR})\), and leptin did not reverse this effect (0.45 \pm 0.08). Using the ACTH assay, the profile of immunoreactive peptides detected after HPLC separation (Fig. 3B) was similar to that seen in the ARC (Fig. 1B). A major immunoreactive peak corresponding to the ACTH (fraction no. 30) peptide was identified together with CLIP (fraction no. 28) and POMC (fraction no. 35). Using the \textit{α}-MSH RIA assay, a prominent peak eluted at the same position (fraction no. 35) was seen for the POMC in the ARC with anti-ACTH (Fig. 3C) but was absent in the ARC with the anti-\textit{α}-MSH (Fig. 1C). To further clarify the nature of this peak we immunoprecipitated fraction 35 with the anti-\textit{α}-MSH antibody, followed by a second reverse-phase HPLC separation. The purified sample was then spotted on an NP20 chip array and read in the Protein Chip system. Analysis of the spectra showed that the molecular weight of this peptide was 28.167 kDa (Fig. 3C, insert).

Analysis of the POMC-derived peptides in the NTS revealed that, in striking contrast to the ARC, the 28.1-kDa peptide, ACTH, and desacetyl \textit{α}-MSH increased during fasting. ACTH levels increased from 0.026 \pm 0.008 to 0.063 \pm 0.006 pg/\mu g protein \((P < 0.05 \text{ vs. CTR})\), but administration of leptin to these animals did not reverse this effect (0.060 \pm 0.005 pg/\mu g protein) (Fig. 3B). Consistent with the increase observed for ACTH during fasting, desacetyl \textit{α}-MSH levels increased from 0.063 \pm 0.004 to 0.122 \pm 0.005 pg/\mu g protein in fasted animals \((P < 0.05 \text{ vs. CTR})\), and administration of leptin did not change the level of desacetyl \textit{α}-MSH (0.103 \pm 0.010 pg/\mu g protein; Fig. 3C). A comparison of the processing products detected in the ARC and in the NTS of fed animals revealed that there was a 23-fold higher (1.044 vs. 45 fmol) amount in POMC peptides produced in the ARC compared with NTS (Table 1). In addition, the processing of POMC in both brain nuclei was different. In both tissues the main product of processing was desacetyl \textit{α}-MSH (95 and 85.7% ARC), indicating that ACTH was mostly converted to desacetyl \textit{α}-MSH by PC2. However, the processing of 28.1-kDa peptide in the NTS was less efficient, since a large amount of this form was detected in this nucleus (Fig. 3C).

In the ARC, we showed that a decrease in the biosynthesis of a POMC during fasting was associated with a decrease in the level of PC1/3. Therefore, we investigated whether this type of regulation could also occur in the NTS. Using the immunohistochemistry protocol, Fig. 4, A and B, depicts a set of representative NTS images stained with anti-\textit{α}-MSH antibodies showing staining in fibers located in the commissural part of the NTS. These fibers cross over below the area postrema and are probably originated from POMC neurons also laterally located within the NTS. We could not see immunostaining in neuronal bodies under these experimental conditions, possibly because we did not use colchicine, which is a required treatment to see immunostaining in cell bodies (35). PC1/3 staining was located in other subnuclei of the NTS and in other regions, including raphe pallidus, lateral reticular, external cuneate, medullary reticular, and gracile nucleus. PC2 was seen in the same regions and also in the dorsal motor nucleus of the vagus.

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**Fig. 2.** Immunolocalization of \textit{α}-MSH, prohormone convertase (PC)1/3, and PC2 in the ARC in fed vs. fasted animals. Coronal hypothalamic sections were obtained from fed (A, D, and G) and fasted (B, E, and H) rats and subjected to immunohistochemistry using anti-\textit{α}-MSH (C), anti-PC1/3 (F), and anti-PC2 (I) antiserum. Each high magnification picture has an inset depicting low magnification from the area. Scale bars: 50 \mu m (low magnification), 20 \mu m (high magnification). AUI, arbitrary unit of intensity.
nerve and in the hypoglossal nucleus. Semiquantitative analysis of immunopositive cells for α-MSH revealed no differences between fed and fasted. PC1/3 and 2 was distributed within the NTS (Fig. 4, D and G). Comparison of the arbitrary units of OD showed that, during fasting, there were no statistical differences in AUI for PC1/3 (Fig. 4F); however, a substantial decrease in PC2 levels was observed (Fig. 4I) in the fibers contained within the NTS. Western blot analysis and RT-PCR for the PCs in this area of the brain stem did not show changes between fed and fasted (data not shown), most likely because the use of a larger region of the brain is analyzed.

DISCUSSION

The central melanocortin system is one of the best-characterized neuronal pathways involved in the regulation of energy balance. The POMC-derived peptide α-MSH is well known for inhibiting food intake and increasing energy expenditure (11, 16). Since melanocortin-3/4 receptor (MCR) antagonists completely blocked the autonomic, satiety, and metabolic effects of leptin, it is believed that the melanocortin system mediates several central actions of leptin (8, 11). This system is particularly important since mutations in MCRs, POMC, or PCs have been associated with obesity in human and rodents (6). Therefore, a better understanding of the melanocortin physiology might contribute to the future development of antiobesity drugs.

Most studies involving the hypothalamic POMC expression have focused on gene expression analysis. However, several aspects of the biology of POMC at the protein level are poorly known. In the ARC, for example, POMC neurons express the leptin receptor ObRb (31), and it is well known that low leptin signaling (e.g., fasting) directly inhibits pomc gene expression and that administration of leptin can attenuate this response (5, 43, 48). Our study corroborated these findings on POMC expression. We also showed a decrease in all POMC-derived peptides, including desacetyl α-MSH. Consistent with this
observation, Pritchard et al. (40), using a specific two-site immunoradiometric assay, found that hypothalamic POMC is decreased during fasting in rats. They did not find changes in the hypothalamic content of ACTH or α-MSH after 48 h of fasting. This discrepancy with our results might be due to differences in the duration of the fasting or to the fact that we worked specifically with the ARC as opposed to the whole hypothalamus. Our data showed that fasting-induced changes in the ARC were more significant for ACTH than for desacetyl α-MSH. This could suggest that the proposed decrease in POMC biosynthesis during fasting is associated with an accumulation of desacetyl α-MSH. Similar conclusions were suggested by the fact that POMC and ACTH levels are reduced in cerebrospinal fluid during fasting and that this decrease was more pronounced than in hypothalamic extracts (40). Additionally, local increased levels of β-MSH in some hypothalamic areas of food-restricted rats have suggested a blockade of peptide release from terminals (23, 50). In addition, a significant fasting-induced decrease in both basal and KCl-stimulated release of γ-MSH peptide was also shown in vitro; however, this study failed to show an acute effect of leptin on the release of γ-MSH (3). Since the leptin administration in fasted rats prevented the fasting-induced decrease in the content of all POMC-related peptides, our results strongly show that leptin potently regulates the biosynthesis of POMC in the ARC. We did not observe an increase in acetyl α-MSH due to the leptin action on the yet undefined N-acetyltransferase activity reported recently (22), which is consistent with the work done in several laboratories (23, 50). We conclude that, under our experimental conditions and with our most sensitive assay for α-MSH (1 pg/tube), leptin is not involved in the N-acetylation of hypothalamic α-MSH.

The POMC-producing neurons in the NTS send projections primarily within the DVC and to other structures in the brain stem and medulla (35, 36). The area studied here and designed as NTS contains mainly POMC products generated within the brain stem since it excludes the parabrachial nucleus, locus coreuleus, and most parts of the paragigantocellular reticular nucleus, which are recipients of fibers from both ARC and NTS (27, 39). The lateral reticular nucleus and the rostral NTS itself (partially contained in our samples) also receive dual innervations; however, the contribution from the ARC seems

Table 1. Distribution of POMC-derived peptides in the ARC and in the NTS in fed animals

<table>
<thead>
<tr>
<th>POMC-Related Peptide</th>
<th>ARC</th>
<th>% †</th>
<th>NTS</th>
<th>% †</th>
</tr>
</thead>
<tbody>
<tr>
<td>POMC</td>
<td>6.3±0.8</td>
<td>0.6</td>
<td>0.9±0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>ACTH</td>
<td>37.0±4.0</td>
<td>3.5</td>
<td>5.6±0.8</td>
<td>12.3</td>
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<tr>
<td>α-MSH</td>
<td>1,001.0±43.4</td>
<td>95.9</td>
<td>39.0±2.0</td>
<td>85.7</td>
</tr>
<tr>
<td>Total</td>
<td>1,044.3</td>
<td>100</td>
<td>45.5</td>
<td>100</td>
</tr>
</tbody>
</table>

Values in fmol/mg are means ± SE. POMC, proopiomelanocortin; ARC, arcuate nucleus; NTS, nucleus of the solitary tract. α-MSH values were calculated using the α-MSH RIA. ACTH and POMC values were calculated using ACTH RIA assay. *Statistical values in fmol/mg of total protein for each peptide; †% amount for each peptide within its own brain region.

Fig. 4. Immunolocalization of α-MSH, PC1/3, and PC2 in the NTS in fed vs. fasted animals. Coronal brain stem sections were obtained from fed (A, D, and G) and fasted (B, E, and H) rats and subjected to immunohistochemistry using anti α-MSH (C), anti-PC1/3 (F), and anti-PC2 (I) antiserum. Each high-magnification picture has an inset depicting low magnification in that area. Scale bars: 50 μm (low magnification), 20 μm (high magnification).
to be minimal, since it has been established by neuronal fiber tracing studies (27, 53). We propose, based on our results, that the biosynthesis of POMC is unique in the NTS and different from the ARC. We showed for the first time the presence of a 28.1-kDa peptide in the NTS; this polypeptide is efficiently processed by trypsin treatment to generate several immunoreactive peaks with the ACTH assay (not shown). However, no visible ACTH was detected, probably because other enzymes such as carboxypeptidases are also necessary (data not shown). The glycoprotein POMC varies significantly in its molecular weight depending upon the levels of O- and N-linked glycosilation (10, 46). Liotta et al. (30) showed by SDS gel electrophoresis that in the hypothalamus there are two forms of POMC with apparent molecular weights of 35 and 31.5 KDa. Since it is a known fact that changes in glycosilation could affect the antigenicity of proteins, and since we do not know whether the POMC-like peptides seen in the NTS and the POMC in the ARC have the same or different types of glycosilation, we speculate that the α-MSH assay could react in a different way with these peptides. This could be the case seen in this study, where the 28.1-kDa peptide in the NTS was detected in fraction no. 35 of the HPLC run (Fig. 3C) but not in the ARC sample (Fig. 1C). That is the reason why we decided to calculate POMC values in Table 1 by using ACTH RIA values, which detect a peak at fraction no. 35 in both samples. The data from Table 1 also showed that there were higher percentages of POMC and ACTH produced in the NTS compared with the ARC. This strongly indicates that the processing of POMC is more efficient in the ARC. However, further studies will determine the biochemical differences between POMC-derived peptides in the ARC and NTS and the implication of this differential processing in the physiology of these POMC neurons. Despite this, to our knowledge this is the first data suggesting that the proteolytic processing of POMC in the NTS and in the ARC is different.

The following evidence suggests that the melanocortin system in the NTS could play a role on the regulation of the energy balance: 1) the administration of melanocortin agonists or antagonists directly in the DVC reduces or increases, respectively, the food intake (52); 2) melanocortin agonists injected in the fourth ventricle increased uncoupling protein-1 expression in the brown fat of rats (51); and 3) melanocortin-4 receptor is highly expressed in brain stem regions such as the DVC (29). However, it is unclear whether the POMC neurons in the NTS are regulated by changes in the energy balance. Our data indicate that fasting alters the biosynthesis of POMC-derived peptides in the NTS, although biosynthesis is not directly regulated by leptin. Recently, it was shown that fasting-induced reduction of pomc mRNA in the in the NTS was not reversed by leptin (26). These findings support our transcription data. The ObRb is present in the DVC, mostly in the DMX and area postrema and in nearly one-third of the neurons of the NTS (20, 47). Also, the marker of leptin action, phosphorylated signal transducer and activator of transcription-3 (pSTAT3), is found in the neurons of the NTS of leptin-treated rats (25, 32). According to these data, delivery of leptin into the DVC decreases food intake and body weight (20). However, our results strongly indicate that this effect of leptin is not mediated by POMC-related peptides produced in the NTS. Different from our study and Huo et al. (26), another laboratory suggested that peripheral administration of leptin induced pSTAT3 activation in about 30% of the POMC-EGFP neurons in the NTS (12). Although the phosphorylation of STAT3 is valid and widely used to indicate leptin signaling, pSTAT3 could participate in other signaling pathways independent of the biosynthesis of POMC. The best way to prove this regulation is by measuring the actual peptide, which was not affected by leptin treatment (Fig. 3).

Similarly to our previous studies (42), where we showed that leptin regulates PCs in the hypothalamic paraventricular nucleus, we found that fasting or low leptin levels resulted in a decrease in PC1/3 in the ARC and a decrease in PC2 in the NTS. At present, it is unknown whether this selective effect of fasting on the PCs seen in both brain regions may contribute to the changes in POMC processing. Nevertheless, these results are consistent with the role that the PCs play to ensure adequate processing of newly synthesized POMC. In maintaining a proper enzyme-substrate homeostasis. Supporting the hypothesis that physiological changes can induce changes in the PCs in turn will affect POMC, a recent study (24) showed that PC1/3 and PC2 gene expression and processing of the prohormone was affected in a coordinate fashion during photoperiod changes in seasonal Siberian hamsters to control body weight. Therefore, the coupled regulation POMC/processing enzymes may be a common process by which cells generate a more effective processing of the prohormone into mature peptides. Based on the current study, Fig. 5 depicts a model of processing in the NTS compared with ARC.

Fig. 5. Schematic representation of POMC posttranslational processing in the ARC as compared with the proposed POMC processing in the NTS. The size of the rectangles represent the amount increased or decreased during fasting. Arrows indicate the site where the PCs produced their cleavages. Dotted lines indicate very low levels of acetyl α-MSH.

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In conclusion, our studies suggest that fasting differentially regulates the biosynthesis and processing of POMC in the ARC and NTS neurons. In the ARC, the fasting-induced downregulation of POMC levels is coupled with the downregulation of PCs. Although the biosynthesis of the POMC-derived peptides in the NTS might also be decreased, these peptides seem to accumulate in this area. Interestingly, leptin upregulates the melanocortin system in the ARC, whereas the POMC neurons of the NTS are not regulated by leptin. Therefore, changes in the nutritional status of the animal affect POMC biosynthesis and processing in a different way in both ARC and NTS regions to accommodate different physiological demands in maintaining proper energy balance.

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